Diglucomelezitose, a Novel Pentasaccharide in Silverleaf Whitefly Honeydew

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Honeydew excreted by the silverleaf whitefly (*Bemisia argentifolii* Bellows and Perring) feeding upon upland cotton (*Gossypium hirsutum* L.) was isolated from cotton lint and fractionated into various oligosaccharides using a charcoal column eluted with aqueous isopropyl alcohol. Two oligosaccharide fractions from the charcoal column, enriched in tetra- and pentasaccharides, were further fractionated on a Bio-Gel P2 column. A fraction from the later column was separated into individual oligosaccharides using high-performance anion-exchange chromatography. One of the sugars isolated in this procedure was found to be novel pentasaccharide. Analysis of this oligosaccharide by NMR showed it to contain sucrose and melezitose moieties. The results of these analyses are consistent with a pentasaccharide of the following structure: *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→3)-*O*-β-D-fructofuranosyl-(2↔1)-*O*-α-D-glucopyranosyl-(4←1)-α-D-glucopyranoside. A tetrasaccharide, maltosucrose [*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside], was also identified in a second fraction from the Bio-Gel column.

Keywords: *Bemisia; carbohydrate; cotton; HPLC; insect*

INTRODUCTION

The silverleaf whitefly, *Bemisa argentifolii* Bellows and Perring, is a considerable pest in subtropical regions. It feeds upon plant leaf phloem sap and excretes a concentrated sugar mixture termed honeydew. This insect can occur in extremely high population densities, resulting in the accumulation of large amounts of honeydew on harvested crops, causing them to become quite sticky and covered with sooty mold. Cotton fiber harvested from silverleaf whitefly-infested fields is frequently contaminated with so much honeydew that it becomes difficult or impossible to process in textile mills.

As a first step in the amelioration of the sticky nature of honeydew-contaminated cotton fiber a study was initiated to identify the sugars in this excreta. Thus far in these studies *Bemisia* honeydew has been found to a complex mixture of 20 or more sugars (Hendrix and Wei, 1994), nearly all of which are created inside the insect (Salvucci et al., 1996) since they do not occur in the insect's diet of phloem sap (Tarczynski et al., 1992). The two most abundant sugar components in this excreta are trehalulose [*O*-α-D-glucopyranosyl-(1→1)-D-fructofuranoside] and melezitose [*O*-α-D-glucopyranosyl-(1→3)-*O*-β-D-fructofuranosyl-(2↔1)-α-D-glucopyranoside]. In addition, this excreta has been found to contain trehalose [*O*-α-D-glucopyranosyl-(1↔1)-α-D-glucopyranoside] and sugars containing this moiety (Hendrix and Wei, 1994; Wei et al., 1996). As in scale insect honeydew (Wolf and Ewart, 1955a), the majority of the sugars in *Bemisia* honeydew appear to be created by transglycosylation reactions involving dietary sucrose. This transfer of glucose moieties from sucrose to the various saccharide acceptor molecules occurs without phosphorylation (White and Maher, 1953).

EXPERIMENTAL PROCEDURES

Materials. A bale of approximately 200 kg of cotton fiber contaminated with *Bemisia* honeydew was washed, 1 kg at a time, in hot deionized water. After removing the fiber by centrifugation, several hundred grams of finely divided (200−400 mesh) activated charcoal was stirred into the water extracts to adsorb the sugars washed off the fiber. The charcoal was next removed from the water by filtration and washed briefly with water. Sugars were then removed from the charcoal by extraction with 95% ethanol. The alcohol in the resulting sugar solution was removed by rotary evaporation under vacuum. The residual water was then removed from the sugars by lyophilization, leaving 1.5 kg of a dark brown syrup.

Methods of Oligosaccharide Purification and Analysis. The oligosaccharides in this syrup were fractionated using a 12.5 × 85 cm charcoal:diatomaceous earth (50:50) column (Whistler and Durso, 1950). Approximately 100 g portions of the sugar syrup were suspended in water, layered on the charcoal column, and eluted with a sequence of 40 L volumes of increasing concentrations (0%, 2%, 4%, and 6%) of isopropyl alcohol in water. The eluate from the 6% propanol elution was collected, and the sugars it contained were isolated by rotary evaporation and lyophilization, as above. A few grams of the resulting sugars were eluted through a heated (Trenel et al., 1969) Bio-Gel P-2 column (4.8 × 120 cm) with deionized water. Sugars in the eluant were detected by passing a portion of the column effluent through a refractive index detector. One of the sugar fractions from the P-2 column which appeared to be enriched in tetrasaccharides was labeled CP-III (Figure 1). A second fraction which appeared to contain mainly pentasaccharides was labeled CP-II.

Fraction CP-III was further fractionated using an NH_2 HPLC column and a complex elution gradient consisting of a

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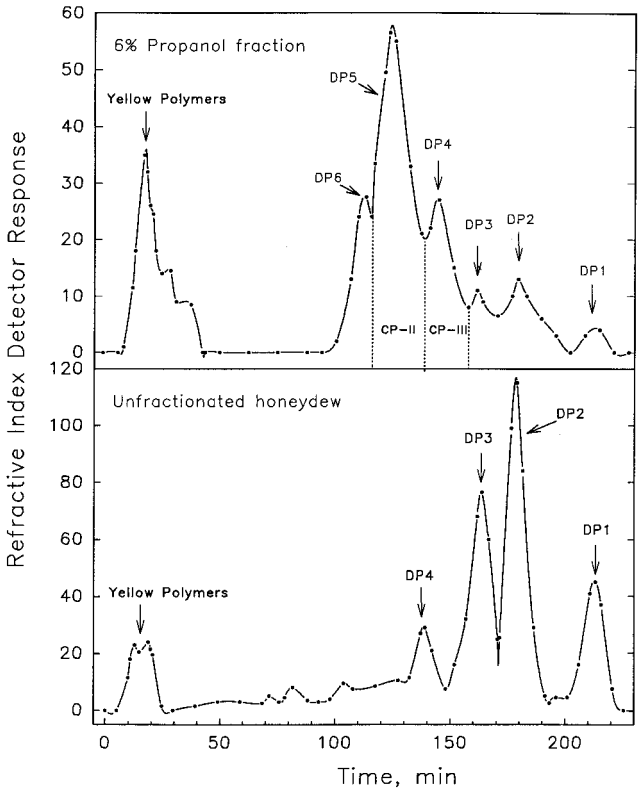

Figure 1. Separation of sugars on a Bio-Gel P-2 column of unfractionated *B. argentifolii* honeydew (lower panel) and a fraction of this excretion eluted from a charcoal column with 6% isopropyl alcohol (upper panel). In the DP*n* labels, *n* represents the number of monosaccharide units in the oligosaccharides in a particular fraction.

Table 1. Gradient for Separation of Fraction CP-III

time (min)	% CH_3OH	% CH_3CN	% H_2O
0.0	8.0	78.0	14.0
5.0	8.0	72.0	20.0
25.0	8.0	69.0	23.0
40.0	8.0	68.0	24.0
48.0	8.0	66.0	26.0
53.0	8.0	78.0	14.0
60.0	8.0	78.0	14.0

Table 2. Gradient for Separation of Fraction CP-II

time (min)	200 mM NaOH	200 mM NaOH + 500 mM sodium acetate
0.0	100.0	0.0
1.0	97.0	3.0
27.0	94.0	6.0
37.0	94.0	6.0
45.0	88.0	12.0
55.0	81.0	19.0
56.0	100.0	0.0
60.0	100.0	0.0

sequence of mixtures of methanol, acetonitrile, and water (Table 1). The sugars which eluted from the NH_2 column were detected with an evaporative light-scattering detector (Wei et al., 1996). Fraction CP-II was further fractionated using two Dionex CarboPac PA-100 HPLC columns operated in series and an eluant consisting of a complex gradient prepared by mixing 200 mM NaOH and 0.5 M sodium acetate in 200 mM NaOH (Table 2). Carbohydrates in the effluent from the CarboPac columns were detected by a pulsed amperometric detector (Hendrix and Wei, 1994). The pH of the effluent from the CarboPac columns was neutralized by a 4 mm Dionex ASRS-I suppression module prior to its entering a fraction collector (Wei et al., 1996). Sodium acetate remaining in the neutralized fractions was removed by elution through the 4.8 × 120 cm Bio-Gel P-2 column with deionized water, and the oligosaccharides were recovered by lyophilization.

Mass Spectrometry. Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF) was applied to an aqueous solution of a native pentasaccharide isolated from Bio-Gel fraction CP-II (CP-II-4) using a desorption matrix described by Mohr et al. (1995). This analysis was performed on a Vestec Lasertec research model instrument operated in the positive ion mode. A nitrogen laser suppling 3-ns pulses of light at 337 nm was used for sample desorption.

Because of its suspected fructose content, a sample of the oligosaccharide CP-II-4 was derivatized for GC/MS analysis under mild reaction conditions. A small amount of inositol (20 μg) was added to the sample as an internal standard. The sample was then reduced with $NaBD_4$. After removal of the borate and sodium, the sample was then methylated using a reagent of 50% NaOH in DMSO. The methylated oligosaccharide was then hydrolyzed under mild acid conditions using 0.1 M TFA at 60 °C for 2 h. After removal of the TFA, the sample was reduced with $NaBD_4$ (10 mg mL^{-1} in 1 M NH_4-OH) for 3 h at room temperature. The reduced sample was then hydrolyzed using 2 M TFA at 121 °C for 2 h. After evaporation of the solvent, the resulting glycosyl residue was again reduced with $NaBD_4$ for 3 h at room temperature and then acetylated using acetic anhydride and pyridine at 121 °C for 20 min. The resulting alditol acetate derivative was dissolved in hexane. These derivatives were then analyzed by GC/MS using a Hewlett-Packard 5985 GC−mass spectrometer and a Supelco SP2330 capillary column (30 m × 0.25 mm).

NMR Spectrometry. A 2.5 mg sample of the pentasaccharide CP-II-4 was dissolved in D_2O (~0.7 mL) for NMR analyses. Spectra were obtained at 25 °C on a Varian Unity-500 spectrometer equipped with a Nalorac 3 mm pulsed-field gradient probe operated at 499.0 MHz for ^1H and 125.7 MHz for ^{13}C (Wei et al., 1996). Chemical shifts were determined with acetone as an internal reference; chemical shift assignments for acetone were 2.224 ppm for protons and 31.00 ppm for the methyl carbons.

Hydrolysis of Oligosaccharides. Two methods of hydrolysis were employed to determine the monosaccharides in these sugars. In the first method a saccharide sample was dissolved in 1 N HCl and the solution was maintained at 105 °C for 1 h, following which it was neutralized with NaOH. In the second method, a mild digestion regime was achieved by hydrolyzing the samples in 1% H_2SO_4 (0.36 N) at 100 °C for 30 min before neutralization of the acid (Basden, 1967). Hydrolysis products from both methods were analyzed by HPLC (Hendrix and Wei, 1994).

Enzymatic Hydrolysis of Oligosaccharides. Small portions of the isolated oligosaccharides were hydrolyzed using 1 mg of invertase mL^{-1} (EC 3.2.1.26; Sigma I-4504, 825 units mg^{-1}) 0.1 M pH 6.0 citrate buffer. These oligosaccharides were also digested, following invertase treatment, with a preparation consisting of 2.3 mg of α-glucosidase mL^{-1} (EC 3.2.1.20; Sigma G-6136, 75 units mg^{-1}) pH 7.0 MOPS (3-[*N*-morpholino]-propanesulfonic acid)−KOH buffer. Both enzymatic digestion regimes were carried out by incubating 200 μL of a 1.0 mg mL^{-1} solution of the oligosaccharide with the enzymes in a 38 °C water bath for 60 min and then quenching the reaction by placing the tubes into a boiling water bath for 6 min.

RESULTS AND DISCUSSION

Figure 1 shows the fractionation of *Bemisia* honeydew and the 6% propanol fraction of honeydew from the charcoal column on a Bio-Gel P-2 column eluted with water. From this figure it is evident that compared to the unfractionated honeydew the 6% propanol fraction is considerably enriched in sugars larger than trisaccharides. Much of the yellow color and stickiness associated with the unfractionated honeydew was absent in the 6% propanol fraction. The remainder of the yellow material which eluted from the charcoal column

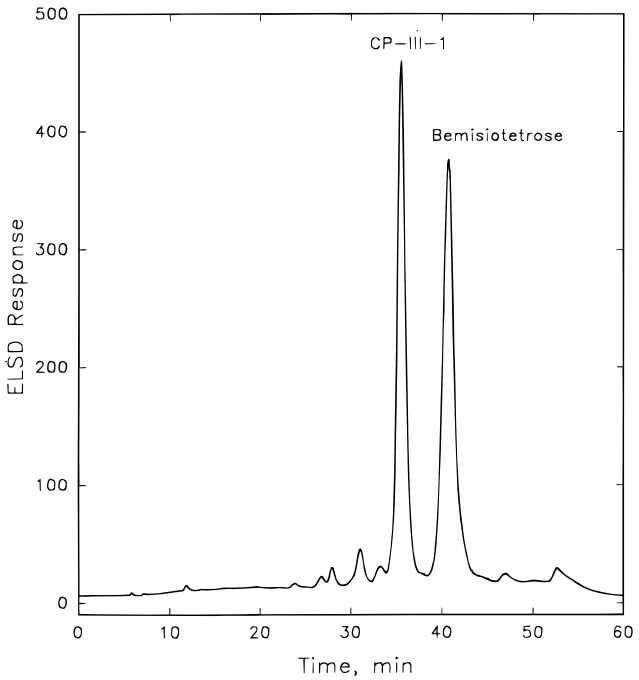

Figure 2. Separation of sugars in fraction CP-III (Figure 1) using an NH_2 HPLC column and an eluant consisting of a sequence of mixtures of H_2O, CH_3CN, and CH_3OH (Table 1). Eluted saccharides were detected by an evaporative light-scattering detector (Wei et al., 1996).

with the 6% propanol fraction was separated from the oligosaccharides in this fraction by the Bio-Gel P-2 column.

Two fractions of the 6% propanol fraction in the P-2 eluate were collected for further characterization. The first of these, which was labeled fraction CP-III, appeared to contain tetrasaccharides (Figure 1). A second fraction, labeled CP-II, appeared to contain mainly pentasaccharides. The oligosaccharides in these two fractions were recovered from the eluant by lyophilization.

Identification of Oligosaccharide CP-III-1. The fraction labeled CP-III was further analyzed by amino column HPLC, and the result of this separation (Figure 2) showed that this fraction consisted primarily of two oligosaccharide peaks, one of which was labeled CP-III-1. This peak constituted about 2% of the total sugars in this honeydew. A second saccharide peak in the HPLC analysis of CP-III was tentatively identified by its retention time as bemisiotetrose, a tetrasaccharide recently reported in *Bemisia* honeydew (Wei et al., 1996). Chromatography of CP-III-1 after its complete acid hydrolysis produced a pronounced peak of glucose and a relatively small peak of fructose. HPLC analysis of the mild acid hydrolysis products of CP-III-1 revealed glucose, fructose, maltose, and maltotriose. Enzymatic hydrolysis of CP-III-1 with invertase released fructose and maltotriose. Incubation of CP-III-1 with a mixture of invertase and α-glucosidase yielded glucose and fructose in the approximate ratio of 3:1. These data are consistent with CP-III-1 being the tetrasaccharide maltosucrose [*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1↔2)-*β*-D-fructofuranoside].

To confirm this tentative identification, several milligrams of CP-III-1 were purified by HPLC and analyzed by NMR. In Figure 3 the ^{1}H NMR spectra of erlose [*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1↔2)-*β*-D-fructofuranoside] and CP-III-1 are compared. The similarities in these spectra clearly indicate a close structural relationship between these oligosaccharides. The tentative identification of CP-III-1 as maltosucrose was confirmed by a careful assignment of the peaks in its ^{13}C and ^{1}H NMR spectra making use of the published ^{13}C spectra of erlose (Munksgaard, 1981; Bock et al., 1984). To assist in the assignment of NMR peaks of maltosucrose, especially its proton resonances, a table of peak assignments for the NMR spectra of erlose was also prepared (cf. Supporting Information).

Some of the signals resulting from the terminal glucose residue in maltosucrose [e.g., the H-2 in the glucose b ring (G_b) at 3.62 ppm] could be distinguished by comparing the NMR spectra of maltosucrose to that from erlose. The published ^{13}C NMR spectrum of erlose was used to locate the corresponding erlose proton signals in its 2D HMQC (heteronuclear multiple quantum correlation) spectra. Peaks in the erlose HMBC spectra were then cross-referenced to the HMQC spectra of erlose to confirm these NMR peak assignments. This cross referencing was very helpful in identifying the erlose H-6 proton signals. After the erlose NMR peaks were assigned, the signals of the carbons of the erlose moiety in the NMR spectra of maltosucrose were identified. These partial assignments provided a very good basis for assigning the remaining peaks. The remaining proton and carbon signal assignments for maltosucrose were then completed by carefully analyzing its 2D COSY (correlated spectroscopy) and TOCSY (total correlation spectroscopy) spectra for proton and carbon correlations. The fine spin coupling structure of the protons in these spectra was determined by means of a 2D homonuclear (H−H) *J*-resolved spectrum.

The presence of maltosucrose in this excreta is somewhat surprising. According to the literature, the oligosaccharides in honey and honeydew are created from sucrose by glycosyltransferase reactions in which glucose moieties are transferred one at a time from sucrose to various acceptor molecules (Duspiva, 1953; White and Maher, 1953). This would suggest that maltosucrose is created by transglycosylation reactions transferring glucose to erlose (glucosucrose), but erlose is not found in *Bemisia* honeydew (Hendrix and Steele, 1991). Erlose, but not maltosucrose, has been reported in bee honey (Goldschmidt and Burkert, 1955), and both erlose and maltosucrose have been reported in the honeydew from certain scale insects (Wolf and Ewart, 1955b; Stephen, 1959).

Identification of Oligosaccharide CP-II-4. Since an HPLC analysis of this fraction indicated it contained numerous oligosaccharides (Figure 4), it was further fractionated using a complex NaOH-sodium acetate gradient (Table 2) and two Dionex PA100 columns in series. We selected one of the oligosaccharides in the PA100 eluate which constituted about 1% of the total honeydew for further characterization. This saccharide was labeled CP-II-4 (peak number 4 in Figure 4).

The eluant used in this chromatography was quite alkaline. To prevent alkali-catalyzed rearrangement or degradation of the separated oligosaccharides, the entire eluant was neutralized immediately after passing through the detector cell by passing it through a Dionex Anion Suppressor Module. Eluant containing the various sugar peaks collected from the anion suppressor was lyophilized to yield white powders. The powder resulting from the lyophilization of peak CP-II-4 was redissolved in deionized water and desalted by elution though a Bio-Rad P-2 gel column with deionized water.

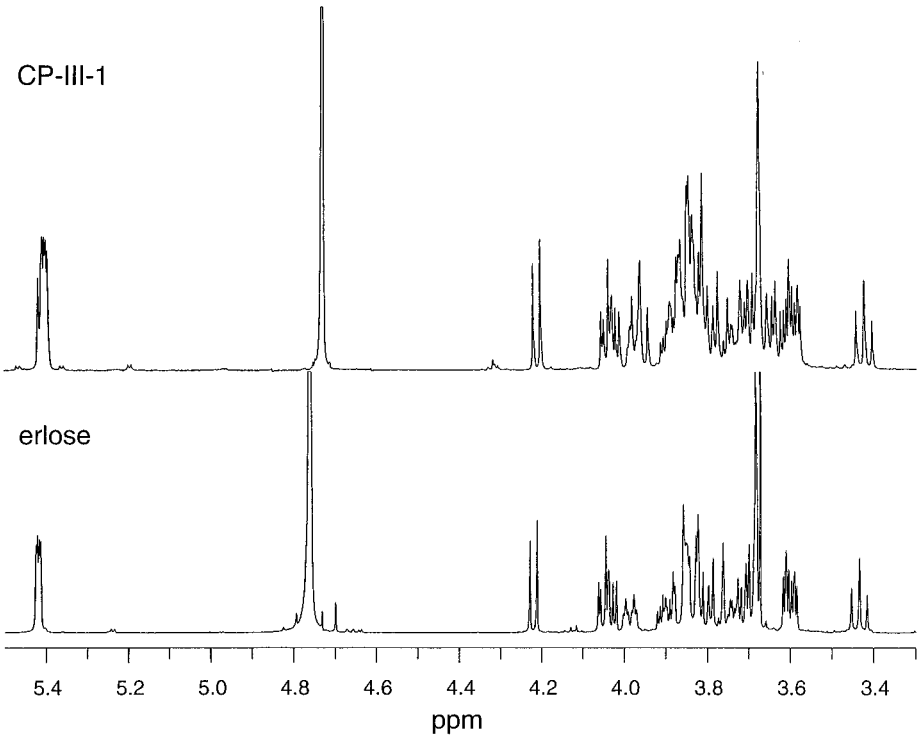

Figure 3. Upper panel: ^{1}H NMR spectra of maltosucrose (CP-III-1). Lower panel: ^{1}H NMR spectra of erlose.

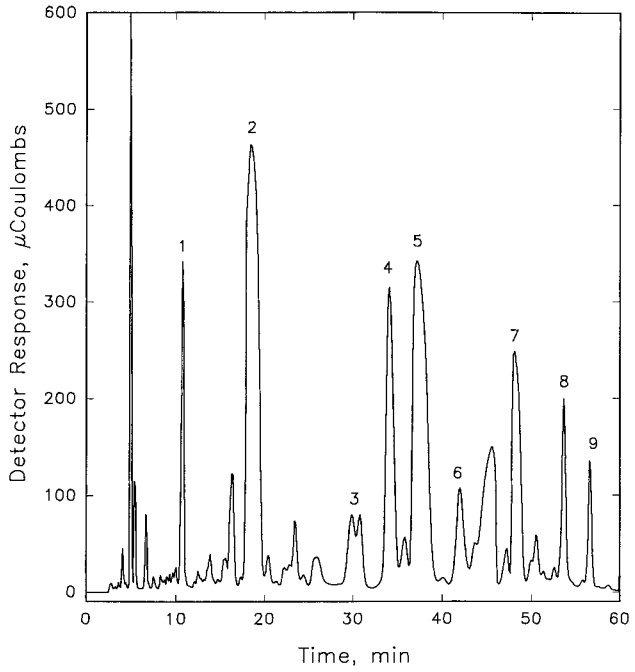

Figure 4. Fractionation of CP-II (Figure 1) by anion-exchange high-performance liquid chromatography on two Dionex PA100 columns and an eluant consisting of a sequence of mixtures of 200 mM NaOH and 0.5 M sodium acetate (Table 2). The peak labeled 4 was determined to be diglucomelezitose (CP-II-4).

Purified CP-II-4 was analyzed by MALDI-TOF. The resulting spectra showed two peaks at *m/z* 868.5 and 852.3, which corresponded, respectively, to the potassium and sodium ion adducts of this compound. These data indicated that CP-II-4 had molecular weight of 828, suggesting it was a pentasaccharide. Total acid hydrolysis of CP-II-4 released glucose and fructose in the approximate ratio of 4:1. GC/MS analyses following methylation indicated that this pentasaccharide contained only terminally linked and 4-linked glucose (t-Glc:4-Glc = 1:1). In this analysis no fructose residues were apparent, possibly because fructose in the permethylated product was destroyed during hydrolysis. However, a mild acid hydrolysis procedure was employed in this analysis which has been successfully applied to other fructose-containing oligosaccharides (Carlson, 1996).

NMR data obtained from CP-II-4 clearly indicated that it was a pentasaccharide. Its ^{13}C NMR spectra exhibited five anomeric carbon resonances (104.42, 101.20, 101.15, 100.33, and 92.53 ppm). Careful analysis of the 2D COSY and TOCSY spectra of CP-II-4 allowed us to trace all the connectivities of the protons from the each sugar ring and to correlate them to the corresponding carbon signals in the HMQC spectra. These data were then cross-referenced to the HMQC and HMBC plots to confirm the tentative peak assignments and to reveal interglycosidic correlations in this sugar. The structural information discovered in the analysis of these NMR spectra from CP-II-4 strongly supported the conclusion from experiments involving the HPLC analysis of digestion products of this oligosaccharide that there is a fructose residue in this molecule which was in the form of a β-furanosyl ring. The NMR data upon which this conclusion was based included the appearance of ^{13}C NMR signals at δ 104.4 (C-2); 83.27 (C-3); 82.05 (C-5); 74.22 (C-4); 62.91 (C-6); 62.68 ppm (C-1). Furthermore, there were clear cross-peaks in the HMBC spectra between the C-2 and the C-3 of the fructose ring and the anomeric protons of two glucose rings. This indicated that the fructose residue in CP-II-4 was substituted at both carbon two and carbon three with glucose moieties which are labeled G_{2a} and G_{3a} in Figure 6. These two glucose units and a fructose moiety linked in this fashion within CP-II-4 constitute melezitose. The existence of melezitose within CP-II-4 was confirmed by comparison of these NMR spectra to the previously published NMR data of melezitose (Anteunis et al., 1975; Bock et al., 1984; Mäler et al., 1995). However, several significant dif-

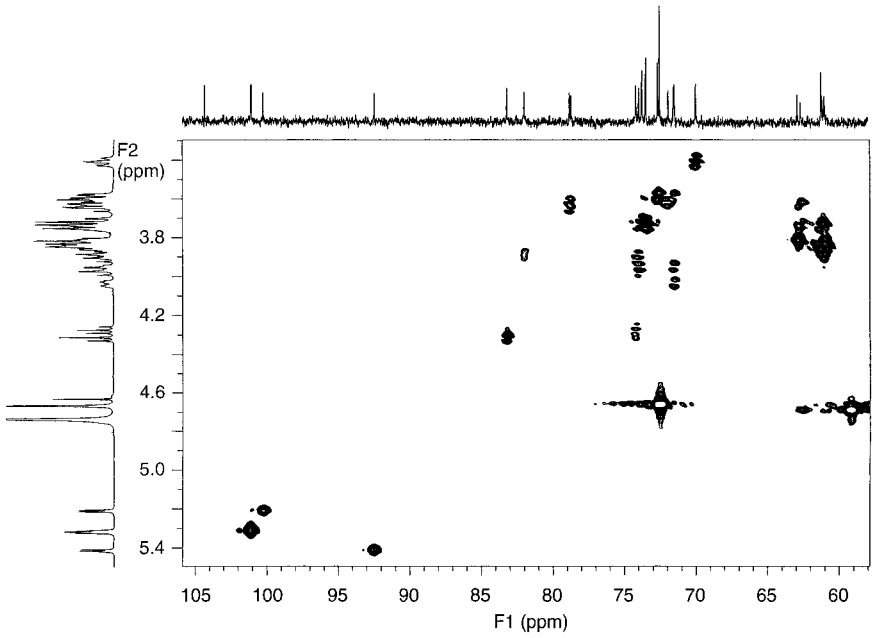

Figure 5. HMQC spectra of diglucomelezitose (CP-II-4).

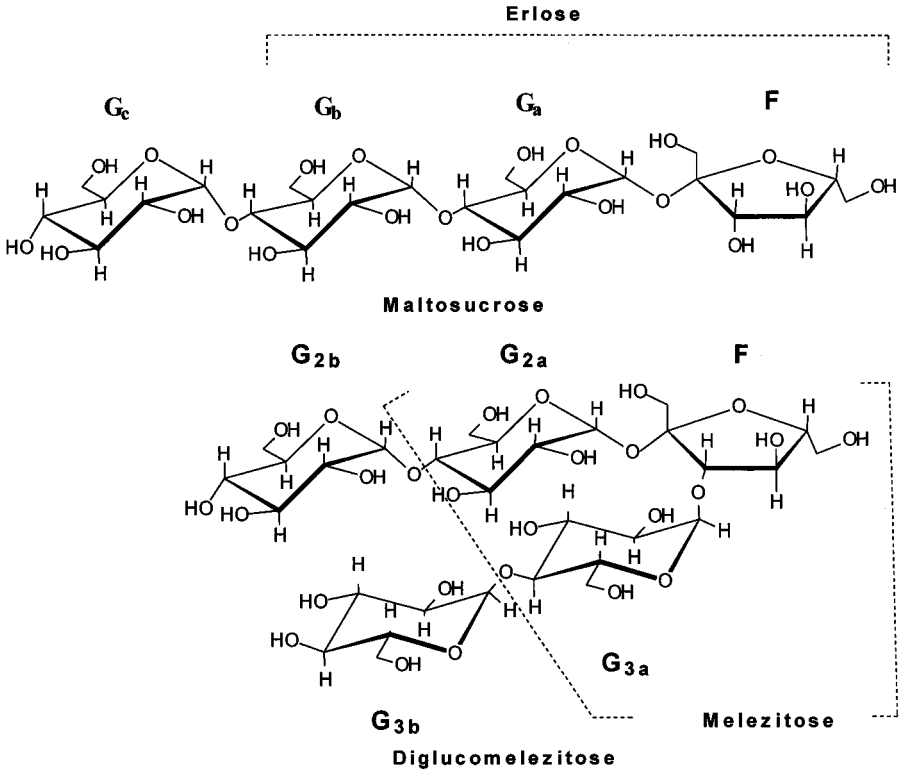

Figure 6. Structures of diglucomelezitose and maltosucrose. The erlose moiety within maltosucrose and the melezitose moiety within diglucomelezitose are indicated.

ferences were found between the NMR spectra of CP-II-4 and that of the corresponding glucoses in melezitose. In the COSY and TOCSY spectra of CP-II-4, the proton coupling network of the G_{2a} and G_{3a} glucose residues showed that both residues are substituted at carbon four. The corresponding ^{13}C signals of C-4-G_{2a} and C-4-G_{3a} (at δ 78.75 and 78.87) in the HMQC spectra confirm this substitution (Figure 5). Therefore, the sugars labeled G_{2a} and G_{3a} in Figure 6 correspond to the two 4-linked glucoses in the methylation analysis. The remaining two glucoses are the terminal glucose residues, one of which is linked to the C-4 carbon of G_{2a} and the other linked to the C-4 carbon of G_{3a}. A proposed structure of CP-II-4, consistent with these data, is given in Figure 6. Based upon this structure we assigned a trivial name of diglucomelezitose to this pentasaccharide.

Diglucomelezitose is structurally very similar to cryptose, a pentasaccharide containing melezitose which is found in the honeydew of the scale insect *Eriococcus coriaceus* which lives almost exclusively on eucalyptus trees (Basden, 1971). In cryptose, two terminal glucose moieties are connected to the third carbon of each glucose in melezitose by α-1,3 bonds, whereas in diglucomelezitose the corresponding glucose units are connected to the fourth carbon of the glucose moieties of melezitose by α-1,4 bonds (Figure 6). Because of the similarity of these two oligosaccharides and the related-

ness of their biological sources (both insects are phloem-feeding members of the Homoptera), we obtained some *E. coriaceus* honeydew from insects living on *Eucalyptus globulus* in New South Wales, Australia, and analyzed it by HPLC using the same elution gradient used earlier to detect diglucomelezitose (Hendrix and Wei, 1994; Figure 4). In this HPLC analysis cryptose and diglucomelezitose were found to elute with significantly different retention times. Because of this difference in retention times and the NMR data mentioned previously, it was concluded that cryptose and diglucomelezitose indeed have different structures.

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Supporting Information Available: Tables of ^{13}C and ^{1}H NMR peak assignments for maltosucrose (CP-III-1), erlose, and diglucomelezitose (CP-II-4) (3 pages). Ordering information is given on any current masthead page.

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